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PRINCIPAL INVESTIGATOR: Suzanne Ostrand-Rosenberg, Ph.D.

CONTRACTING ORGANIZATION: University of Maryland
Baltimore County
Baltimore, Maryland 21228-5398

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Suzanne Ostrand-Rosenberg, Ph.D.

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For many patients with mammary cancer the primary tumor can be successfully treated by surgical removal, however the long-term prognosis is not favorable because of the high frequency of metastatic disease which is not treatable by current approaches. Our goal is to develop vaccination strategies to minimize metastatic disease. We postulate that an improvement in the generation of mammary carcinoma-specific $CD4^+$ T helper lymphocytes will facilitate development of CD8-mediated tumor immunity. To enhance the activation of $CD4^+$ T helper cells, autologous mouse mammary tumor cells have been transfected with syngeneic MHC class II genes plus costimulatory and antigen presentation accessory molecules, including B7-1, B7-2, IL-1α, and the superantigen, SEB. The genetically modified tumor cells should be able to directly present mammary tumor peptides to $CD4^+$ T cells, thereby facilitating T cell activation. Transfectants are being tested for their tumorigenicity, for their ability to protect tumor-free mice from subsequent exposure to mammary tumor cells, and for their ability to vaccinate against spontaneous metastatic disease.

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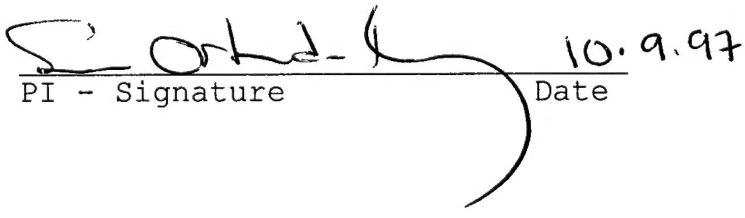

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(5) INTRODUCTION

For many patients with mammary cancer the primary tumor can be successfully treated by surgical removal, however the long-term prognosis is not favorable because of the high frequency of metastatic disease which is not treatable by current approaches. We are using tumor-specific immunotherapy to curtail the incidence of metastatic breast cancer. Some of the most efficient anti-tumor mediators are tumor-specific $CD8^+$ T lymphocytes. In most cases, for optimal activity $CD8^+$ T cells require "help" from antigen-specific $CD4^+$ T lymphocytes (1-3). Recent studies indicate that the inability of the tumor-bearing host to reject tumors may be due to a lack of adequate tumor-specific T_h lymphocytes (3-6) We have therefore hypothesized that tumor-specific T_h activity can be significantly improved by generating tumor cells that contain all of the necessary antigen presentation, accessory and costimulatory molecules such that they are competent for tumor peptide presentation to $CD4^+$ T cells, and thereby facilitate T_h cell activation (reviewed in (7)). Such genetically engineered tumor cells could be used as vaccines to prevent development of metastatic breast cancer, and thereby enhance a host's tumor-specific immune response.

Our strategy is to genetically modify tumor cells so that they can directly present mammary carcinoma tumor peptides to $CD4^+$ T helper cells, thereby by-passing the requirement for professional antigen presenting cells and making more efficient the presentation of tumor peptides to T helper lymphocytes (reviewed in (7)). Accordingly, in the *first specific aim* we are using DNA-mediated gene transfer techniques to generate mammary tumor cell transfectants expressing many of the molecules constitutively expressed by professional antigen presenting cells (APC). These molecules include the peptide binding structures or MHC class II molecules, as well as several costimulatory molecules which have been shown to deliver the requisite second signal for T cell activation. The costimulatory molecules to be used include: B7-1 (8, 9), B7-2 (10) and 4-1BB ligand (11-13). 4-1BB ligand is a very recently described costimulatory molecule that is expressed by professional APC such as macrophages and B lymphocytes. Binding of 4-1BB ligand to its counterreceptor 4-1BB on $CD4^+$ and $CD8^+$ T cells transmits a potent costimulatory signal to the T cells resulting in T cell activation. Since 4-1BB ligand appears to function independently or synergistically with other costimulatory molecules (11) it appears to be an excellent candidate for coexpression with B7 genes for enhancing tumor-specific immunity. Mammary tumor cells expressing the cytokines IL-1(14) and IL-12 (15, 16) potent inducers of T_{h2} and T_{h1} lymphocytes, respectively, are also being generated. In addition, the gene encoding the bacterial superantigen, SEB, a potent polyclonal T cell activator (17), is being transfected into the mammary tumor lines.

In the *second specific aim* we are determining the tumorigenicity of the transfectants, and their ability to protect the syngeneic host against subsequent challenges of wild type tumor. We will also determine the ability of the transfectants to "rescue" mice carrying established wild type mammary tumors, and identify the helper and effector lymphocytes functional in mammary tumor rejection. In the *third specific aim* we are determining if metastatic mammary cancer can be reduced or prevented by immunization or concomitant treatment with the tumor cell transfectants. This novel tumor-specific immunotherapy approach should significantly improve the host's immune response to autologous breast tumor, and may provide several potential strategies for immune intervention in metastatic mammary cancer.

(6) BODY

During the 1st and 2nd years of this grant (Aug. 1994 - July 1996) we established the mouse mammary tumor system and PCR cloned the MHC class II, B7.1, and 4-1BBL costimulatory molecule genes needed for the proposed studies. These earlier studies proceeded slowly because our initial experiments were performed with genes obtained from other labs, and many of these genes were faulty (see year 1 Annual Report). By the end of the 2nd year of the grant (August 1996), we had generated various mammary tumor cell transfectants expressing syngeneic MHC class II or B7.1 genes, and had begun to test their tumorigenicity in syngeneic female BALB/c mice. During the 3rd year of the grant (Sept. 1996-August 1997), we have further refined the mouse 4T1 mammary tumor system and have tested the MHC class II and B7.1 transfectants as immunotherapeutic agents for mice with established metastases. Also during the 3rd grant year, we have resumed the studies using IL-1 transfected tumor cells, since new observations have allowed us to use a form of IL-1 (IL-1α) that is less toxic to the host. We have also added an additional gene to our bank of transfectants, the gene encoding the superantigen, SEB, because SEB is known to be a powerful polyclonal T cell activator. Many of these transfectants have impressive immunotherapeutic activity as measured by their ability to significantly reduce or eliminate established 4T1 mammary tumor metastases.

The BALB/c-derived 4T1 mouse mammary tumor is an excellent animal model for human breast cancer. During grant year 2 we demonstrated that within 18 days of inoculation into the mammary fat pad, the 4T1 tumor spontaneously metastasizes to the draining lymph node, lungs, liver, and blood. We have now extended these observations, and assessed spontaneous metastasis to the central nervous system (brain). As shown in **table 1**, the 4T1 tumor also metastasizes to the brain, although this is a less frequent event than metastasis to other sites.

Table 1 also demonstrates that close to 100% of mice develop lung metastases, and that these metastases are detectable as early as two weeks after inoculation of primary tumor.

Since extent of metastases in patients is frequently related to the size of the primary breast tumor, we have also assessed the number of metastatic cells in mice carrying primary 4T1 tumors of various sizes. As shown in **figure 1**, there is a rough correlation between diameter of the primary 4T1 tumor and number of lung

Harvest Day	Spontaneous Metastases				
	Lymph Node	Lung	Liver	Blood	Brain
14-18	11/12 (2-57)	13/13 (1-43)	0/11	0/13	ND
22	6/8 (5-35)	6/11 (32-338)	3/5 (1)	1/8 (1)	ND
30-32	2/3 (15-83)	10/10 (6-116,500)	7/8 (7-3,700)	3/4 (6-82)	ND
34-37	ND	10/12 (315-267,000)	9/11 (32-7,300)	5/11 (1-24)	3/11 (1-116)
>42	ND	14/14 (1109-200,000)	6/8 (1,100-12,200)	6/8 (25-490)	4/6 (5-613)

BALB/c mice were challenged s.c. in the abdominal mammary gland with $5x10^3$ parental 4T1 tumor cells. Mice were sacrificed at various times after tumor challenge and the draining lymph node, lung, liver, blood, and brain tissues were removed. Data indicate the number of animals positive for spontaneous metastases out of the total number tested for each organ type. The numbers in parentheses shows the range of clonogenic metastases found in the positive organs. ND, not done.

Table 1: 4T1 mammary carcinoma cells spontaneously metastasize in BALB/c mice. Mice were challenged s.c. in the mammary fat pad with $5X10^3$ 4T1 tumor cells, sacrificed at various intervals, and draining lymph node, lung, liver, blood, and brain analyzed for number of metastatic cells. Numbers in ( ) are the range of clonogenic cells.

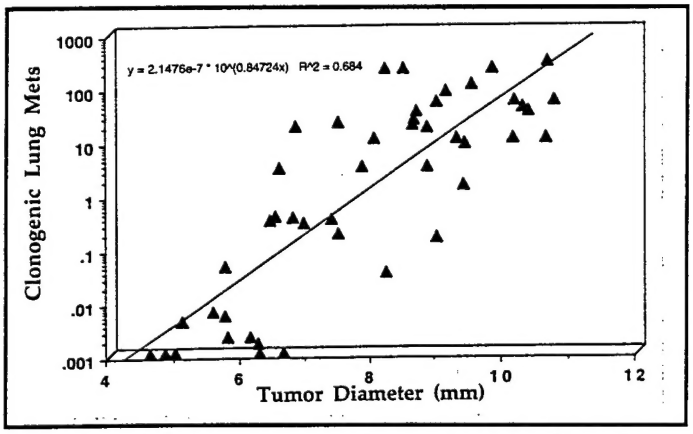

Figure 1: 4T1 mammary carcinoma metastasis to the lung is directly proportional to primary tumor size. Female BALB/c mice were injected s.c. in the mammary fat pad with $5X10^3$ 4T1 cells, sacrificed at various intervals, and the number of metastatic tumor cells in the lungs determined. Each point represents an individual mouse.

metastases. Based on its origin, ability to spontaneously metastasize, site and kinetics of metastasis formation, the 4T1 mouse tumor closely models human breast cancer, and, therefore, is an excellent animal model for the studies presented below.

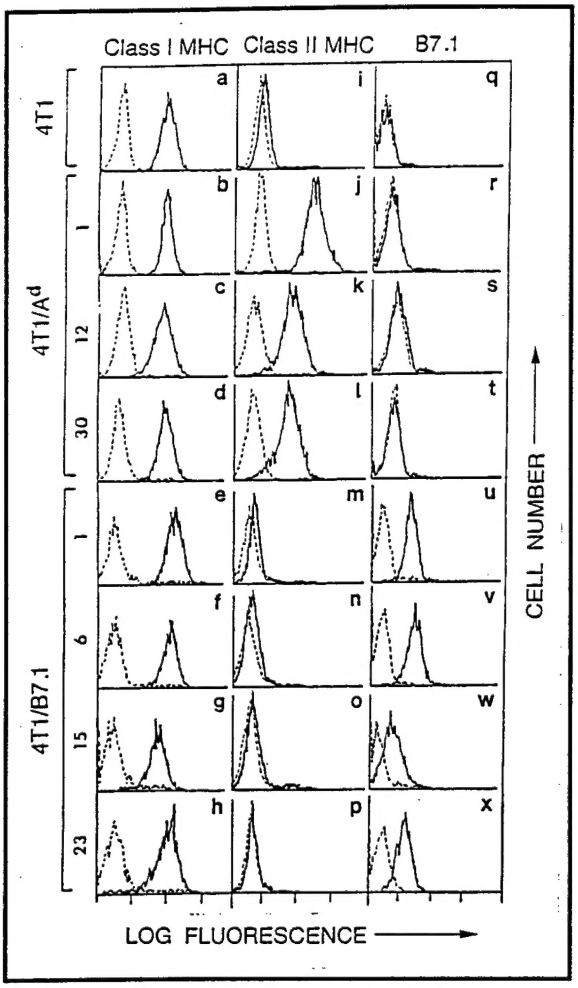

Figure 2: 4T1 mammary tumor cell transfectants express I-A^d or B7.1. Transfectants were stained by indirect immunofluorescence for MHC class II (I-A^d; MKD6 mAb), MHC class I (H-2D^d, 34-5-8 mAb); or B7.1 (1G10 mAb).

in the following studies. As previously described, 4T1 tumor cells were transfected with syngeneic MHC class II (A_α^d and A_β^d genes) or B7.1 gene. Several clones of each transfectant line were selected. As shown in **figure 2** the selected clones have varying levels

4T1 transfectants expressing syngeneic MHC class II and costimulatory B7.1 molecules have been generated. Although some flow cytometry data were presented in the 2nd year report, we now present a full analysis of the 4T1 transfectants used

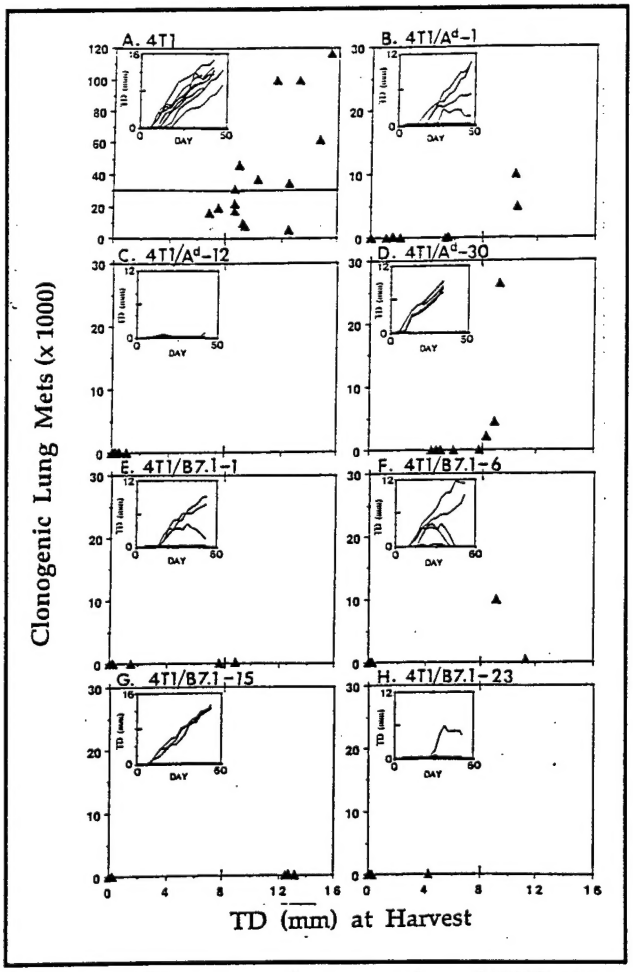

Figure 3: 4T1 tumor cells expressing of MHC class II or B7.1 have reduced metastatic potential. Female BALB/c mice were injected s.c. in the mammary fat pad with 5X10^3 4T1 cells or transfectants as indicated. 32 to 55 days later, the number of metastatic cells in the lungs was determined (large panels). Growth of primary tumor was also monitored (small, inset panels).

of expression of the respective transgenes. The transfectants, therefore, express the desired genes, and can be tested as immunotherapeutic agents.

MHC class II and B7.1 transfected 4T1 cells have reduced metastatic potential. In the 2nd year report we presented data documenting primary tumor formation of 4T1/A^d and 4T1/B7.1 transfectants in immunocompetent BALB/c mice. These clones have now been tested for metastasis formation. As shown in **figure 3**, most of the transfectants are significantly less metastatic than wild type 4T1 tumor transfectants in immunocompetent BALB/c mice, even though primary tumor formation is comparable to wild type tumor. In T cell deficient BALB/c *nu/nu* mice (**figure 4**), 2 of the clones are as metastatic as wild type 4T1 cells, while 2 clones show reduced metastasis formation. Expression of MHC class II or B7.1, therefore, reduces the metastatic potential of 4T1 cells, and this reduction may be due to a T cell-mediated response.

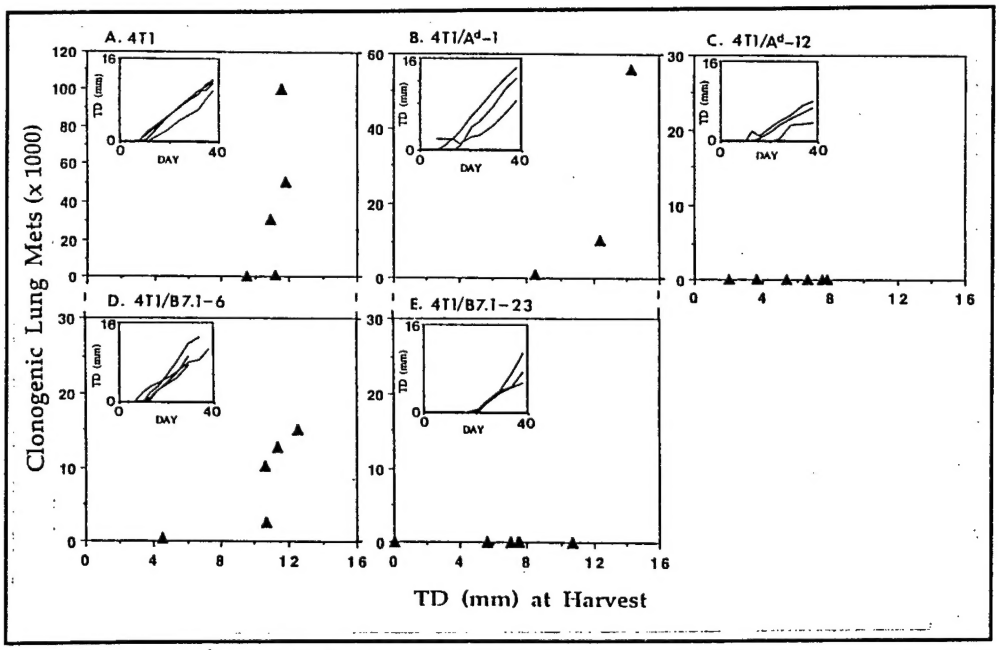

Figure 4: 4T1/A^d and 4T1/B7.1 transfectants have mixed growth patterns in BALB/c *nu/nu* mice. Tumor cell inoculations and analyses were performed as in figure 3, except BALB/c *nu/nu* mice were used.

Immunization of naive mice with 4T1 transfectants significantly reduces subsequent metastatic disease. Since our goal is to produce cell-based vaccines that protect against metastatic disease, we have tested the MHC class II and B7.1 transfectants for their ability to immunize tumor-free mice. Immunocompetent,

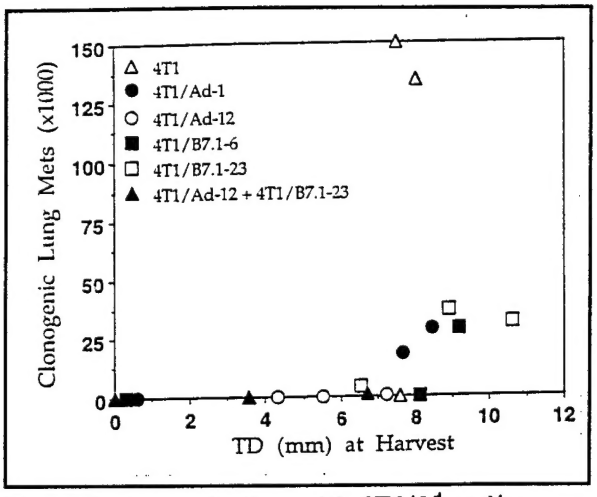

Figure 5: Immunization with 4T1/A^d and/or 4T1/B7.1 transfectants protects naive mice against metastatic disease. BALB/c mice were immunized with the indicated cells/transfectants and challenged 4 weeks later in the mammary fat pad with 5X10^3 wild type 4T1 tumor. Metastases were analyzed as per fig. 1.

syngeneic BALB/c mice were immunized with irradiated 4T1/A^d, 4T1/B7.1, or a mixture of 4T1/A^d +4T1/B7.1 cells, and challenged 4 weeks later with live, wild type 4T1 tumor. As shown in **figure 5,** mice immunized with any of the transfectants had significantly fewer lung metastases than the control 4T1 immunized mice, and mice immunized with the 4T1/A^d-12 clone had even fewer metastases. The transfectants, therefore, are effective cell-based vaccines for protecting tumor-free mice against later challenges of wild type tumor.

Treatment of tumor-bearing mice with 4T1/A^d and/or 4T1/B7.1 transfectants reduces established metastatic disease, but does not affect primary tumor growth. Because of the strong immunization effect of the transfectants in tumor-free mice, the transfectants have also been tested as immunotherapeutic agents in mice carrying established 4T1 tumors. Immunocompetent BALB/c mice were inoculated s.c. in the mammary fat pad with 5X10^3 4T1 cells, two weeks later treated with the various transfectants, and 4 weeks after treatment, lungs were removed and assayed for metastatic tumor cells. As shown in **figure 6,** mice treated with either an MHC class II transfectant (4T1/A^d) or a B7.1 transfectant (4T1/B7.1) have significantly reduced metastases relative to mice treated with unmodified 4T1 cells. However, mice treated with the mixture of 4T1/A^d + 4T1/B7.1 cells have the greatest reduction in metastatic cells. Since our earlier studies (see **table 1**) demonstrated that lung metastases are already present 2 weeks after 4T1 inoculation, immunotherapy with genetically modified 4T1 tumor cells is an effective treatment for reduction of established metastases.

Administration of MHC class II and B7.1 transfected 4T1 cells combined with systemic IL-12 significantly reduces spontaneous metastatic disease. IL-12 is known to facilitate the development of T_{h1} lymphocytes (15). Since our goal is to improve tumor-specific T_{h1} differentiation, we have combined our cell-based

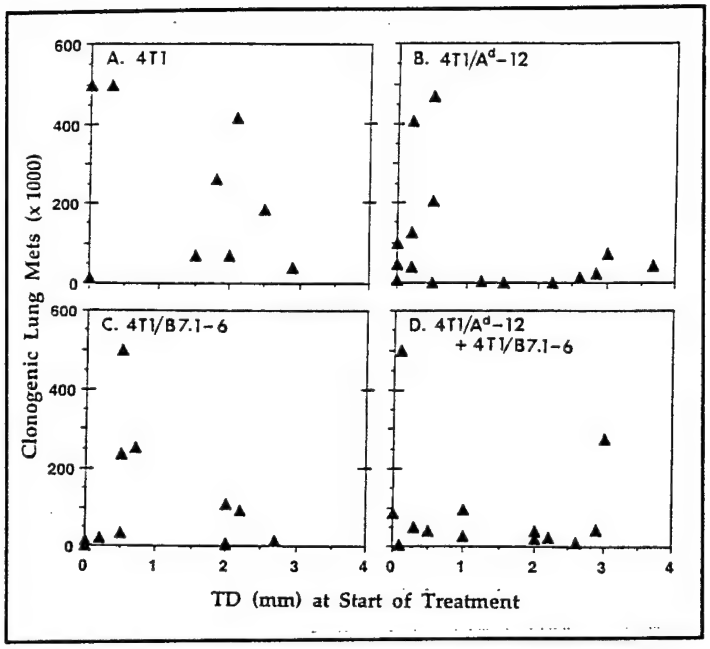

Figure 6: Immunotherapy of mice with established 4T1 tumors with 4T1/A^d and/or 4T1/B7.1 transfectants reduces metastatic disease. BALB/c mice were inoculated s.c. in the mammary fat pad with 5X10^3 4T1 cells, two weeks later treated with the indicated 4T1 transfectants, and 4 weeks later lungs removed and assayed for metastasis formation.

vaccines with administration of systemic IL-12 for the treatment of mice with 3 week established metastases. BALB/c mice were inoculated s.c. in the mammary fat pad with 5X10^3 wild type 4T1 cells, and 3 weeks later started on the following course of treatment: Once a week 10^6 irradiated 4T1, 4T1/A^d and/or 4T1/B7.1 cells i.p.; 3 times a week 1 μg rIL-12 i.p. After 3 weeks of treatment, mice were sacrificed and the number of metastatic cells in the lungs determined. As shown in **figure 7**, mice treated with rIL-12 plus a mixture of 4T1/A^d and 4T1/B7.1 cells had significantly fewer metastatic cells than other treated mice, although IL-12 alone also had a significant effect on reducing metastatic disease. Since mice with 3 week established tumors have significant metastatic loads, this combination treatment is quite effective in reducing formation of new metastases and/or reducing existing metastatic disease.

4T1 tumor cells transduced with a retrovirus carrying the IL-1α gene have reduced tumorigenicity. In our original grant we proposed to assess the effects of IL-1β expression as a costimulatory molecule. Experiments with IL-1β transfected sarcoma cells, however, indicated that IL-1β secreted from the transfectants was toxic to the hosts (see previous Annual Report). Use of IL-1, therefore, required a form of the molecule that would retain its costimulatory functions, but not be toxic. We reasoned that the IL-1β transfectants were cytotoxic because IL-1β is a secreted molecule and when the transfectants were inoculated into mice, high levels of systemic IL-1β accumulated. To overcome the toxicity, but maintain the costimulatory effects, we sought a membrane-bound form of IL-1. As a result, we have established a collaboration with Dr. R. Apte of the Beer Sheva University in

Israel. Dr. Apte has developed a retrovirus containing the IL-1α gene (18). IL-1α is similar to IL-1β in its costimulatory properties, however it is principally a membrane-bound or cytoplasmically expressed molecule (19).

4T1 tumor cells were transduced with supernatant from an IL-1α secreting fibroblast cell line provided by Dr. Apte, and transductants selected for G418 resistance (the retrovirus contains the neo^R gene). Lysates of the resulting cells were screened by ELISA for IL-1α activity. As shown in **table 2**, as compared to non-transduced 4T1 cells, the 4T1/IL-1α transductants express IL-1α.

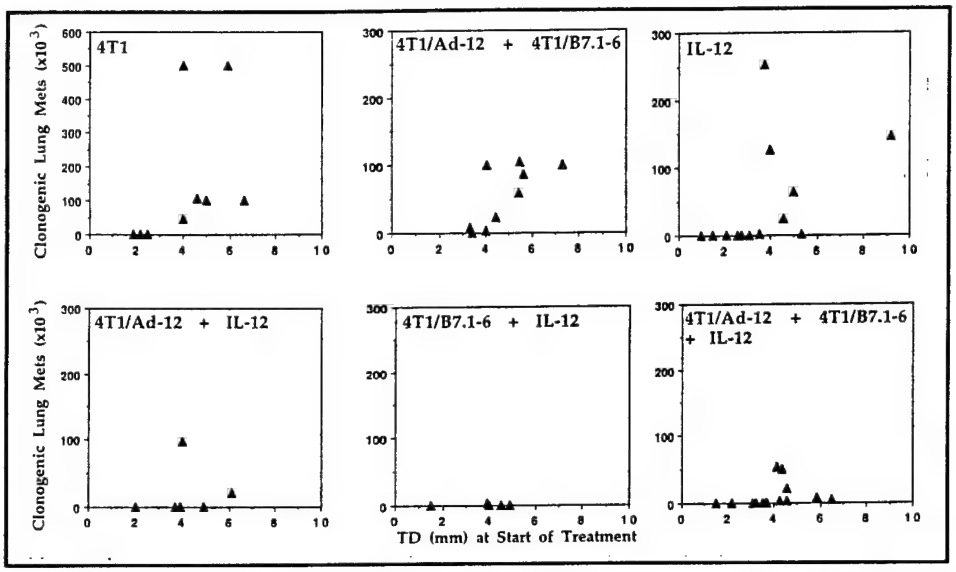

Figure 7: Treatment of mice with established 4T1 metastases with MHC class II and B7.1 transfected 4T1 cells plus systemic IL-12 causes significant reduction in metastatic disease. BALB/c mice with 3 week established spontaneous metastases were treated as described in the text. At completion of the immunotherapy protocol, lungs were assayed for number of metastatic cells. Each point represents an individual mouse.

Cells	IL-1α (ng/ml ± SD)		
4T1	0.71 ± 0.01	1.2 ± 0.2	0.20 ± 0.05
4T1/IL-1α	4.0 ± 0.04	7.0 ± 0.0	3.3 ± 0.54

Table 2: 4T1/IL-1α transductants express IL-1α. Lysates of 10^6 4T1 and 4T1/IL-1α tumor cells were assayed by ELISA for IL-1α expression. 3 independent experiments are shown.

To test the tumorigenicity of the 4T1/IL-1α transductants, female BALB/c mice were inoculated with $5X10^3$ control 4T1 and 4T1/IL-1α tumor cells and primary tumor formation and metastasis outgrowth determined. As shown in **figure 8**, wild type 4T1 cells formed primary tumors and grew progressively, while 4T1/IL-1α tumor cells did not form tumors. Likewise, wild type 4T1 cells were metastatic to the lungs, while 4T1/IL-1α cells did not metastasize (**table 3**). Expression of tumor cell encoded IL-1α, therefore,

eliminates the tumorigenicity and metastatic potential of 4T1 mammary carcinoma cells.

4T1 tumor cells transfected with the superantigen, SEB, are less tumorigenic than wild type 4T1 tumor cells.

Superantigens, such as SEB, are polyclonal activators of T lymphocytes and have been shown to activate tumor-specific T cells (17). We have, therefore, reasoned that 4T1 tumor cells expressing the SEB gene should be effective cell-based vaccines for activating tumor-specific T lymphocytes.

The bacterial SEB gene was subcloned into the pHβ-Apr-1-neo expression vector under control of the human β-actin promoter (20) and the resulting plasmid used to transfect 4T1 tumor cells. Transfectants were screened for SEB expression by RT-PCR and cell extracts were tested for T cell proliferation activity in a mixed lymphocyte reaction (MLR). As shown in **figure 9**, supernatants from 4T1/SEB clones 12 and 14 secrete approximately 20 μg/ml SEB/10^5 cells/3 days, indicating expression of the SEB transgene. Tumorigenicity of the 4T1/SEB clones was tested by challenging syngeneic BALB/c mice with $5X10^3$ tumor cells and monitoring primary tumor growth. As shown in **figure 10**, the 4T1/SEB transfectants are significantly less tumorigenic than wild type 4T1 cells, suggesting that the 4T1/SEB transfectants may be effective immunotherapy agents.

Tumor Cells	Spontaneous Metastases		
	Lung	Blood	Liver
4T1	3/4 (0->10^5)	0/2	2/3 (14-39)
4T1/IL-1α	0/4	0/4	0/4

Table 3: 4T1/IL-1α tumor cells are not metastatic. BALB/c mice were inoculated s.c. with $5X10^3$ 4T1 or 4T1/IL-1α cells, sacrificed 6 weeks later, and the number of metastatic cells in the lung, blood and liver determined. Fractional numbers indicate the number of mice with metastases; numbers in ( ) indicate the range of clonogenicmetastatic cells per mouse.

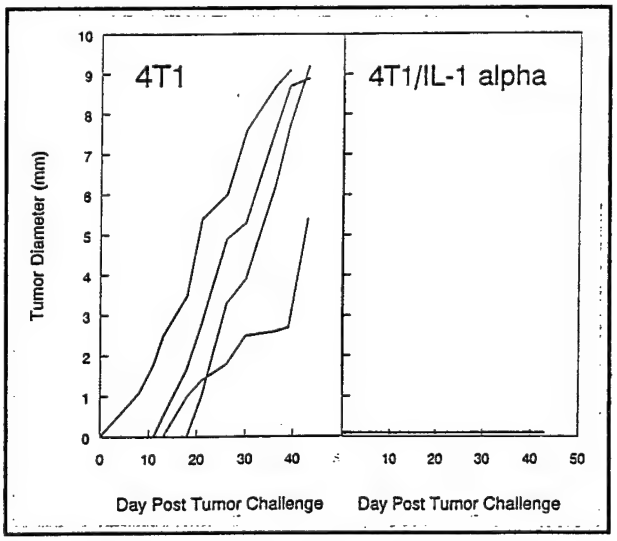

Figure 8: 4T1/IL-1α tumor cells do not form primary tumors in vivo. BALB/c mice (4 mice per group) were inoculated s.c. with $5X10^3$ 4T1 or 4T1/IL-1α cells and primary tumor growth monitored.

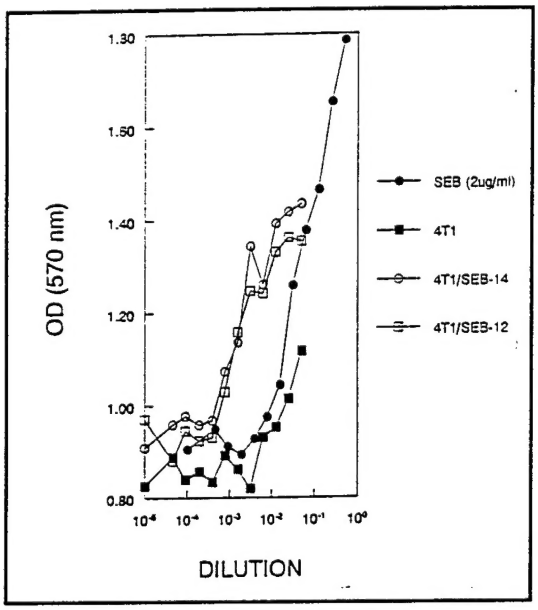

Figure 9: 4T1/SEB transfectants secrete SEB. 4T1 and 4T1/SEB cells were cultured at 10^5 cells/ml/3 days and the supernatants tested for T cell proliferative activity on syngeneic BALB/c splenocytes. Proliferation was measured by MTT assay and read at OD 570 nm. Soluble SEB at a starting concentration of 2 μg/ml was the standard. Log dilutions of the culture supernatants and SEB standard were tested and are plotted on the absicssa. The two 4T1/SEB clones secrete approximately 20 μg/ml SEB.

4T1 tumor cells have been transfected with the gene encoding the 4-1BBL costimulatory gene. During the 2nd grant year we PCR cloned the gene encoding the costimulatory molecule, 4-1BBL (11). During this past year, 4T1 tumor cells have been transfected with the PCR-cloned 4-1BBL gene. These transfectants express 4-1BBL by RT-PCR analysis, however, we have been unsuccessful in detecting 4-1BBL at the protein level. Several approaches have been tried, including staining with a fluorescently tagged 4-1BB ligand, and staining with a fluorescently labelled mAb to 4-1BBL. We have not extensively pursued these experiments because the other transfectants are such promising therapeutic agents, and because we have been kept more than busy working with the other transfectants and transductants. We anticipate that during the final year of this grant we will return to the 4T1/4-1BBL transfectants.

(7) CONCLUSIONS:

During this 3rd grant year we have tested the immunotherapeutic efficacy of many of the transfectants and transductants generated during the first two years of the grant. Several of these cell-based vaccines are very effective immunotherapeutic agents for the treatment of advanced, established, spontaneous metastatic mammary cancer. Unlike most other animal studies, our tumor system closely models human breast cancer. In addition, unlike other studies, we are treating relatively advanced metastatic disease in which tumor-bearers have significant numbers of lung, lymph node, blood, and/or liver metastases. Our results, therefore, may be more generally applicable to clinical situations. Because we see significant reductions, and in some cases elimination, of metastatic disease in treated mice, we view these newly developed cell-based vaccines as very promising immunotherapeutic agents.

We have several goals for the final year of this grant. 1) We will continue the therapy studies and try to develop more effective protocols for reducing metastatic disease. Since the three cell-based vaccines (4T1/A^d + 4T1/B7.1 cells, 4T1/SEB cells, and 4T1/IL-1α cells) most likely mediate their effects through independent T cell activation pathways, we will combine the treatments to optimize therapeutic efficacy. We will also test the transfectants/transductants not already tested in a therapy protocol (4T1/SEB, 4T1/IL-1α), and continue studies with the completely untested 4-1BBL transfectants (4T1/4-1BBL). 2) We will identify the immune cells that mediate metastasis regression via in vivo by antibody depletion studies. 3) We will further refine our therapy protocol so that it more closely models human disease as follows: Mice will be inoculated with wild type tumor in the mammary fat pad and the primary tumor surgically removed at a time when metastases are well established (2-4 weeks after initial inoculation). Immunotherapy will then be started. This protocol will model the clinical situation in which women have established metastases at the time of removal of the primary tumor, and may provide useful information on the clinical efficacy of our newly developed cell-based vaccines.

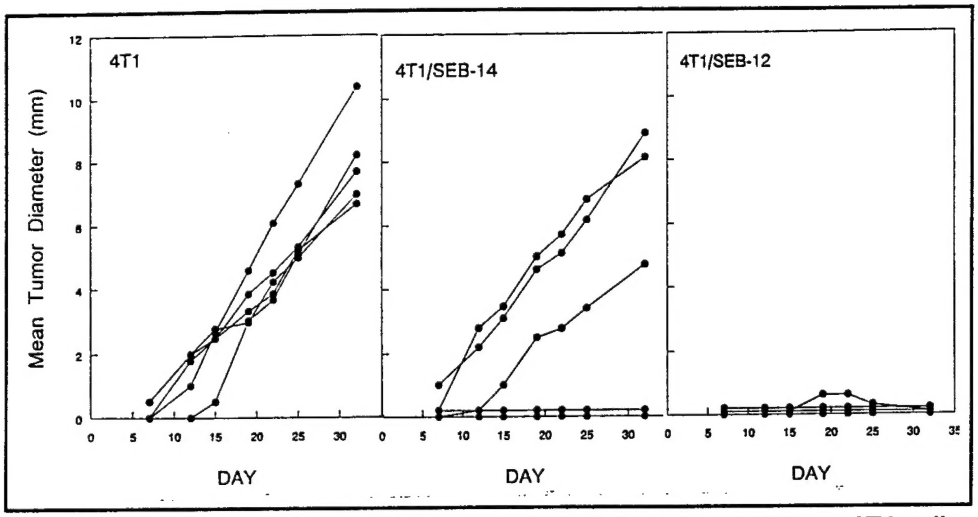

Figure 10: 4T1/SEB transfectants are less tumorigenic than wild type 4T1 cells. BALB/c mice were challenged s.c. in the mammary fat pad with $5X10^3$ tumor cells and primary tumors measured at varying intervals. Each line represents growth of an individual tumor.

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